

Isolation of a Major Cell Surface Glycoprotein from Fibroblasts

(transformation/roteolysis/urea/iodination/gel electrophoresis)

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ABSTRACT A cell surface component has been isolated in partially purified form from cultured chick embryo and chick heart fibroblasts. This glycoprotein is similar to a protein recently reported to be present at the surface of normal cells, and missing after neoplastic transformation. It is a major cell surface glycoprotein that is synthesized by cultured fibroblasts, but is not collagen. It is shown to be markedly trypsin-sensitive, and its recovery from the cell surface is dependent on cell density. It is excluded from Sephadex G-200, but is not rapidly sedimented by ultracentrifugation, and has an apparent molecular weight of 220,000. The isolation of this cell surface glycoprotein may now provide a means of determining its function.

Alterations of cell surface components have been implicated in the loss of growth control in neoplastic transformation. Changes in lectin agglutinability, adenylate cyclase activity, glycosyl transferases, and quantities of various cell surface glycolipids and glycoproteins have been demonstrated (1-8). To forge a causal link between these changes and the altered behavior of transformed cells and to analyze the mechanisms involved will require the isolation and characterization of the pertinent biologically active cell surface components.

Recently, there have been several reports that a cell surface glycoprotein of 200,000-250,000 daltons is present on several "normal" cell types, but is markedly diminished or absent from their transformed variants (9-13). We report the extraction and preliminary characterization of such a protein from chick embryo and chick heart fibroblasts. The conditions of isolation should be gentle enough to permit future evaluation of its biological function.

MATERIALS AND METHODS

Culture Methods. Secondary cultures of 10-day chick embryo fibroblasts were prepared by a modification of the methods of Rein and Rubin (14). After 3-4 days of primary culture, cells were harvested with 0.125% (w/v) trypsin (Difco) plus 10 μ g/ml of DNase (Schwarz/Mann), passed to 100-mm plastic dishes at 5×10^6 per 12 ml of medium, and cultured to a necessary final cell density of about 2.5×10^5 /cm². Primary cultures of minced 7-day chick embryo heart ventricles were established in 25-cm² plastic culture flasks (Falcon). Secondary cultures were prepared at densities of 6 to 8×10^6 per 100-mm dish from the outgrowths after 2 days in culture.

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; NaDodSO₄, sodium dodecyl sulfate; CSP, cell surface protein; MW, molecular weight; PAS, periodic acid-Schiff.

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Culture medium consisted of Dulbecco's modification of Eagle's medium containing 0.3 mg/ml of glutamine, 10% fetal-calf serum (Grand Island Biological), and penicillin and streptomycin (100 units and 100 μ g/ml, respectively). Various labeling procedures used the following radioactive compounds purchased from New England Nuclear Corp.: L-[U-¹⁴C]amino acid mixture; L-[³H]leucine (32.1 Ci/mmol); L-[¹⁴C]leucine (280 mCi/mmol); L-[¹⁴C]proline (255 mCi/mmol); DL-[³H]tryptophan (179 mCi/mmol); and carrier-free Na¹²⁵I.

Isolation of Surface Protein. The following extraction procedure maximized purity and yield of the cell surface protein, and minimized retraction of the cell monolayers. Cultures were rinsed three times with Hanks' balanced salts solution buffered with 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4 (Calbiochem). Cells on 100-mm dishes were then extracted 2 hr at 37° in 5 ml of serum-free Dulbecco's medium with glutamine on a gyratory shaker (model G-2, New Brunswick Scientific) at 30 rpm. This first extract in serum-free medium was discarded, and the monolayers were washed once with HEPES-buffered Hanks' solution. The cells were then further extracted for 2 hr in serum-free medium to which urea (Schwarz/Mann, ultra-pure) was added to a final concentration of 0.2 M immediately before use. This second extract was removed, chilled, and centrifuged at $25,000 \times g$ for 15 min.

Cell loss after this extraction protocol was minimal: <0.8% of the total was found in the pellets, and no loss was detected when parallel dishes before and after extraction were compared (1.4×10^7 /100-mm dish compared with 1.6×10^7 , respectively). Little toxicity was noted at this concentration of urea in long-term cell cultures. Cell proliferation was unaffected during 4 days in culture in 0.2 M urea. Incorporation of [¹⁴C]leucine at 0.1 μ Ci/ml into acid-precipitable radioactivity during the last 24 hr of a 2-day incubation of cells in urea medium was 87% of controls.

Polyacrylamide Gel Electrophoresis. All extracts were dialyzed against distilled water for 48-72 hr and lyophilized in siliconized conical centrifuge tubes. Samples were resuspended in 1% sodium dodecyl sulfate (NaDodSO₄), 10 mM sodium phosphate (pH 7.0), 0.1 M dithiothreitol (Calbiochem), 10% glycerol, and 0.001% bromophenol blue, then placed immediately in boiling water for 3 min. Cell monolayers were homogenized according to Hynes (10) with 2% NaDodSO₄ in phosphate buffer containing 2 mM phenylmethylsulfonyl fluoride (Calbiochem) to inhibit proteolysis, and prepared for electrophoresis as above.

NaDodSO₄-polyacrylamide gel electrophoresis was performed on 5% slab (15) or cylindrical gels. Slab gels were run

in a continuous 0.1% NaDodSO₄, 1 mM EDTA, 25 mM Tris-192 mM glycine (pH 8.3) buffer without a stacking gel (acrylamide and methylene bisacrylamide from BioRad). The cylindrical gels used an 0.5% ethylene diacrylate cross-linker (Borden Chemical Co., Philadelphia) in a 0.1% NaDodSO₄-0.5 M urea system (16, 17). Slab gels were run at 25 mA until the bromphenol blue marker reached the end of the gel (1.5-2 hr); cylindrical gels were run at 12 mA per gel for 4.5 hr. Gels were fixed and stained for protein with Coomassie Brilliant Blue (Schwarz/Mann) by various protocols (18, 19, or 20) without apparent differences in results. Cylindrical gels of radiolabeled samples were frozen unstained or after protein staining. They were sliced, solubilized, and counted as described (17). Slab gels were dried as described (15), and autoradiograms were made on medical x-ray film (Kodak, No Screen). Mobilities were determined relative to bromphenol blue. Molecular weight standards included myosin (200,000; ref. 21), β -galactosidase (130,000; kindly provided by Dr. Frank Reithel), phosphorylase *a* (94,000; Sigma), bovine serum albumin (68,000; Schwarz/Mann), and ovalbumin (43,000; Sigma). When plotted on a semilogarithmic scale, marker protein mobility was linear according to molecular weight in both gel systems. Densitometry was performed with a Grant Instruments (Berkeley) comparator-microphotometer on the absorbance mode, with the dark-yellow filter (about 580 nm). Relative quantities of protein in the gels were estimated and compared by integrating the area under absorbance curves.

Periodic acid-Schiff (PAS) staining for carbohydrate was performed on cylindrical and slab gels (22, 23); NaDodSO₄ was removed by prefixation (24). The electrophoresis standards listed above were routinely included as controls. Weak, variable staining of myosin or other nonglycoproteins was encountered even with shortened periodic acid incubations. Such weak, nonspecific staining of controls necessitates caution until it is possible to obtain sugar analyses of purified cell surface protein (CSP).

RESULTS

Cell Surface Protein. There are more than 50 Coomassie blue-staining bands of apparent molecular weight (MW) >20,000 in gels of cell homogenates (Fig. 1a). Of these, eight bands were labeled with ¹²⁵I by a lactoperoxidase-mediated reaction (Fig. 1d). One heavily labeled band near the top of gels is very sensitive to mild proteolysis (Fig. 1b and e). This fact, plus the demonstration by others (10, 25) that the labeling procedure attaches iodine to accessible tyrosine-containing peptides, supports the notion that this band represents a cell-surface component. This surface protein appears broad, with diffuse boundaries in Coomassie blue-stained gels (Fig. 1a).

Removed by Gentle Procedures. Cell cultures extracted with serum-free medium release proteins that can be concentrated and run on polyacrylamide gels. The major band seen on such gels corresponds to the prominent, diffuse, iodine-labeling, trypsin-sensitive protein band seen in gels of cell homogenates. The yield of this cell surface protein (CSP) can be increased considerably by including 0.2 M urea in the extraction medium. Its purity can be improved by extracting sequentially in serum-free medium, then in medium containing 0.2 M urea (Table 1, Fig. 2). The purified CSP band comigrates with the ¹²⁵I-labeled peak when urea extract and iodine-labeled homogenate are subjected to electrophoresis together (data not shown). This observation further supports the no-

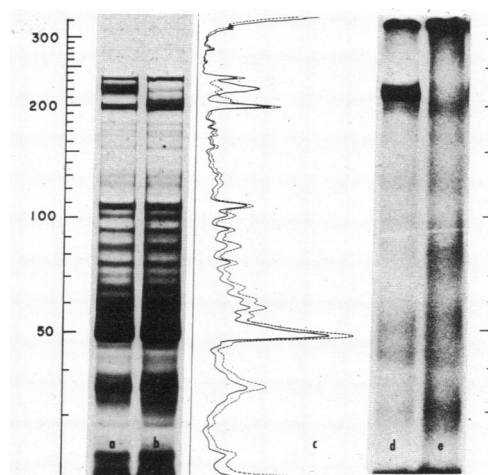


FIG. 1. Trypsin sensitivity and ¹²⁵I-labeling of CSP on intact cells. Chick embryo fibroblasts at about 2.5×10^5 cells per cm² were treated as indicated, then subjected to electrophoresis on NaDodSO₄-5% polyacrylamide slab gels and stained with Coomassie blue. Scales indicate apparent molecular weight ($\times 10^{-3}$). (a) Homogenate of control culture; amount equivalent to 7.5×10^5 cells applied to gel. Note that the band at MW 220,000 is broader and has more diffuse edges in comparison with adjacent bands. (b) Homogenate of parallel culture trypsinized with 1 μ g/ml of trypsin at 37° for 3 min. Note that the diffuse band is no longer present, that a lightly staining pair of thin bands only barely discernible in the control is now visible (apparently representing similarly migrating proteins not accessible to trypsin), and also that a new band of apparent MW about 205,000 has appeared. No other bands seem significantly altered. (c) Superimposed absorbance tracings of samples (a) (solid line) and (b) (broken line). The absorbance of the highest peak was 0.544. (d) Lactoperoxidase-mediated ¹²⁵I labeling of a cell monolayer (10); 50 μ g applied to the gel. Note that the most heavily labeled band on this autoradiogram corresponds in apparent MW and band-width to the trypsin-sensitive band in (a). (e) Monolayer trypsinized (10 μ g/ml at room temperature for 3 min), then labeled with ¹²⁵I. Note that the cell surface protein band is absent, but that other iodinated bands remain.

tion that the extracted protein and the identified surface component seen in cell homogenates are identical.

Probably Glycoprotein. In NaDodSO₄ homogenates of whole cells, the CSP stains with PAS, indicating that it is probably a glycoprotein, as suggested by others (9-13, 29). Isolated CSP also stains with PAS (Fig. 2e). Treatment of cell monolayers or the isolated CSP with low levels of trypsin (0.0001% at 37° for 3 min) results in a loss of the PAS-stained CSP band on NaDodSO₄ gels (Fig. 2d and f).

Large Molecular Weight. The apparent MW of the CSP is 220,000, on both the cylindrical and slab gel systems (assuming a MW of 200,000 for myosin). This value could be an overestimate, since some glycoproteins migrate anomalously in NaDodSO₄ gels (30). However, the identical molecular weight estimates from two systems of differing crosslinking, as well as from 10% slab gels, support this estimate (see ref. 30). The apparent MW was not affected by treatment of the sample with 10 mM EDTA or by reduction for over 1 hr in 0.1 M dithiothreitol at 37°.

The CSP appears in the void volume of Sephadex G-200 columns eluted with phosphate buffer (Fig. 3). Its exclusion from Sephadex G-200, which sieves globular proteins of

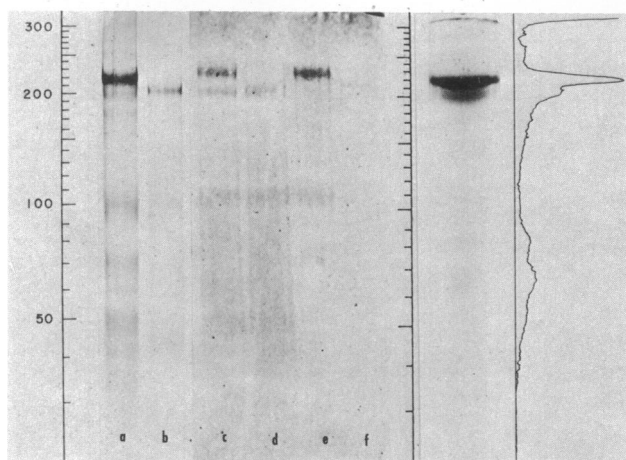


FIG. 2. Trypsin sensitivity and PAS staining of isolated CSP. Two halves of a 5% slab gel stained with Coomassie blue (a,b) or PAS method (c-f). CSP isolated from 6×10^7 chick embryo fibroblasts was pooled, lyophilized, and resuspended in 0.5 mM CaCl_2 , 50 mM Tris-HCl (pH 7.4). Half of the sample was trypsinized (1 $\mu\text{g}/\text{ml}$ for 3 min at 37°) and half served as a control. (a) Isolated CSP. Note that band width and apparent MW are similar to those of homogenates (Fig. 1). (b) Trypsinized isolated CSP. Note that the band is eliminated, that no other comigrating bands are revealed, and that this brief proteolysis results in a less intensely staining band of apparent MW 205,000. This lower MW band corresponds to that seen after brief trypsinization of whole cells (Fig. 1b). (c) Control whole-cell homogenate stained by PAS, equivalent to 7.5×10^6 cells. Note relatively heavily stained CSP band. (d) Homogenate from parallel culture trypsinized as above. Note elimination of CSP band to PAS staining. (e) Isolated CSP, control. (f) Trypsinized isolated CSP. Note that the CSP band is eliminated with the appearance of a weakly staining lower MW band as in (b) and (d). On the right, for comparison, CSP isolated from embryonic chick heart fibroblasts with corresponding absorbance tracing. Samples were subjected to electrophoresis on a NaDodSO₄-5% polyacrylamide cylindrical gel; mobility of the CSP was 0.20 and peak absorbance was 0.544. Lower MW band (shoulder on trace) may also be CSP degradation product. CSP of comparable purity can also be isolated from this cell type grown in a completely different culture medium [Medium 199 plus 2% tryptose phosphate broth, 1% calf serum, and 1% heat-inactivated chicken serum (14)].

5000–800,000 daltons, suggests that it is either a highly asymmetric molecule, or is aggregated. In addition, preliminary ultracentrifugation experiments suggest that isolated CSP sediments as a particle of less than 20S. After centrifugation at $125,000 \times g$ in a Spinco Type 50 angle-head rotor at 4° , both supernatants and pellets were subjected to electrophoresis on 5% gels. Densitometry of the Coomassie blue-stained gels revealed that 74% of the CSP remained in the supernatant after 1.5 hr of centrifugation; 43% after 2.6 hr; and 24% after 5.2 hr. Particles of 20S and larger should have sedimented within 5.2 hr under these conditions (33).

Synthesized by Cells. The CSP peak is the predominant labeled peak on NaDodSO₄ gels of extracts from chick embryo fibroblasts labeled with [¹⁴C]aminoacids (Fig. 4). Chick embryonic heart fibroblast extracts show a similar labeling pattern when incubated 24 hr in 1 $\mu\text{Ci}/\text{ml}$ of [³H]leucine, with the predominant peak corresponding to the CSP (see Table 1). The amino acids, [³H]tryptophan (10 $\mu\text{Ci}/\text{ml}$ for 24 hr)

TABLE 1. Extraction of proteins from chick embryo fibroblast cultures

	Total protein* (μg)	Cell surface protein	
		% Absorbance†	% Incorporation‡
Cell homogenate	2080	2.3 [0.5]‡	nd
First extract	27	7 [9]	nd
Second extract (in 0.2 M urea)	33	18 [23]	24 [32]
Second extract (if urea omitted)	24	9	nd

Combined data from 4 separate experiments; each value is for 1.5×10^7 cells in a 100-mm culture dish. All extracts were centrifuged at $25,000 \times g$ for 15 min.

* Mean of duplicate protein determinations (27). Although protein in extracts from duplicate dishes varied by <20% within the same experiment, values from different experiments could differ by as much as 50%.

† Aliquots of NaDodSO₄ homogenates or resuspended extracts were subjected to electrophoresis on NaDodSO₄-polyacrylamide gels. After samples were stained with Coomassie blue, the percent of total absorbance due to the CSP peak was determined by densitometry. The CSP peak area for homogenates was defined as the difference between control and trypsin-treated samples (Fig. 1c). Peak area was proportional to CSP concentration in a dilution series. However, percent absorbance due to CSP is only a crude approximation of purity since different proteins may bind different amounts of the stain (28). Consequently, the following calculations based on the table are only estimates: Assuming the second extract in urea is 18% pure, the amount of CSP recovered from one dish would be 6 μg . If the CSP represents 2.3% of the total cell homogenate protein, it is at a concentration of 3 pg per cell. Assuming a MW of 220,000 for CSP (see text), there are about 8×10^6 CSP molecules per cell.

‡ Figures in brackets represent comparable values determined from chick heart fibroblast cultures.

§ Chick embryo fibroblasts were labeled 24 hr in a [¹⁴C]aminoacids mixture (Fig. 4). The percent of the total label on NaDodSO₄ gels which was found in the CSP peak was determined. The chick heart fibroblasts were labeled 24 hr in 1 $\mu\text{Ci}/\text{ml}$ of [³H]leucine.

nd = not determined.

and [¹⁴C]proline, are also incorporated into the CSP peak, the latter less efficiently than into procollagen (Fig. 5).

Not Collagen. As reported for other fibroblasts (34, 35), the major proteins incorporating [¹⁴C]proline produced by chick embryo fibroblasts in culture are identified as procollagen on NaDodSO₄ gels (Fig. 5). Collagenase (Worthington, CLSPA, 431 units/mg) eliminates the procollagen protein bands and the corresponding [¹⁴C]proline-labeled peaks. The CSP, however, does not migrate with either of the presumed procollagen $\alpha 1$ or $\alpha 2$ bands, or with rat tail collagen, and is unaffected by collagenase treatment (Fig. 5).

Recovery is Density Dependent. The amount of CSP recovered in the gentle extraction protocol was markedly dependent on cell density. Specifically, the amount of CSP recovered from a constant number of cells (10^7) increased linearly with increasing cell density. In contrast, the amount of CSP in whole-cell NaDodSO₄ homogenates of a constant number of cells did not change significantly with increasing

cell density (Fig. 6). These results suggest that increasing cell interaction may facilitate the release of CSP.

DISCUSSION

A major cell surface protein (CSP) has been isolated from chick embryo and chick heart fibroblasts by sequential extractions with serum-free medium and low concentrations of urea. The procedure recovers about 5-10% of the total amount of CSP from fibroblast cultures in partially purified form. The remainder apparently remains in association with the intact monolayer of extracted cells (unpublished). Since PAS staining of the CSP band on polyacrylamide gels is relatively strong and is trypsin-sensitive, it is likely that CSP is a glycoprotein (see refs. 9-13 and 29, but also the reservations in *Materials and Methods*). The relative broadness of the CSP band on NaDodSO₄ gels could, therefore, reflect varying glycosylation of carbohydrate side chains (36). It could also, however, reflect polypeptide variability, e.g., a class of similar, comigrating proteins, as suggested for the erythrocyte protein, spectrin (37).

Similar concentrations of urea release a factor from chick heart fibroblasts that restores contact inhibition of movement to urea-"transformed" cells (38). However, while such activity can be demonstrated in impure extracts, preliminary experiments have not shown activity in more highly purified CSP preparations.

It seems likely that we have isolated the same high molecular weight surface component of normal fibroblasts which others have shown to be altered after cell transformation (9-13; also see ref. 39). It will be important to establish how the loss of this CSP relates to the loss of growth control and the other accompanying cell surface alterations (1-8) after neo-

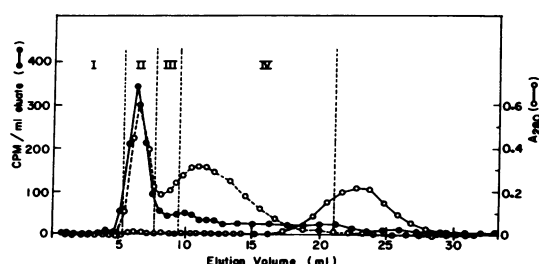


FIG. 3. Exclusion of CSP from Sephadex G-200. All CSP extracts were centrifuged at $125,000 \times g$ for 1.5 hr to remove particulate matter, dialyzed against column buffer (20 mM phosphate, pH 7.0), and concentrated (31). One milliliter was applied to a 1.5×15 -cm column and eluted. O, absorbance. The major absorption peaks are standards: the earliest-eluting absorbance peak is Blue Dextran; the second, unreduced bovine serum albumin; and the third, phenol red. Roman numerals indicate elution fractions, which were dialyzed, lyophilized, and subjected to electrophoresis on a slab NaDodSO₄ gel. The CSP peak of each fraction was quantitated by densitometry and expressed as percentage of the total amount of CSP applied to the column. Fraction I: 0-5.3 ml, 4.8%; II: 5.3-7.5 ml, 75%; III: 7.5-9.5 ml, 10%; IV: 9.5-21 ml, <0.1%. Total recovery was 90%. (●) CSP extract from chick embryo fibroblasts labeled with 1 μ Ci/ml of [³H]leucine for 24 hr. (○) Unlabeled CSP extract, undialyzed (containing 0.0015% phenol red). Note miniscule absorbance peak in void volume (sample estimated to contain less than 10 μ g of CSP). (○—○) Blue Dextran 2000 and bovine serum albumin, 1 absorbance unit/ml. Note that most of the labeled material and most of the electrophoretically determined CSP elutes in the void volume.

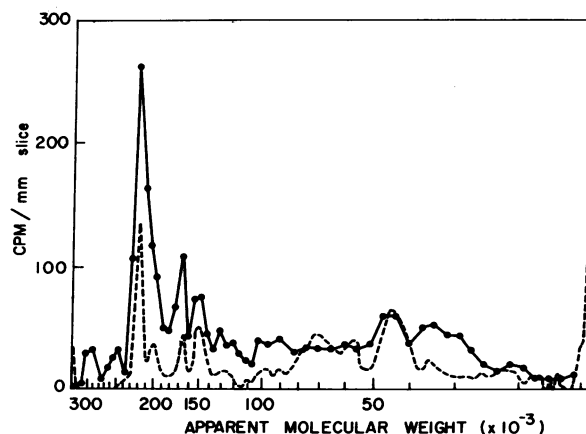


FIG. 4. Incorporation of [¹⁴C]aminoacids into CSP. NaDodSO₄-polyacrylamide electrophoresis of CSP extract from 1.3×10^7 chick embryo fibroblasts grown for 24 hr in the presence of 1 μ Ci/ml of [¹⁴C]aminoacids mixture. After it was fixed and stained (22), the gel was scanned. The same gel was then frozen, sliced, solubilized, and counted in a scintillation counter. (●) counts/min; (broken line) absorbance, in arbitrary units.

plastic transformation. It may be significant that the low levels of protease capable of eliminating this protein by labeling and protein staining criteria (Fig. 2) are similar to those which can induce lectin agglutinability, reduce cyclic

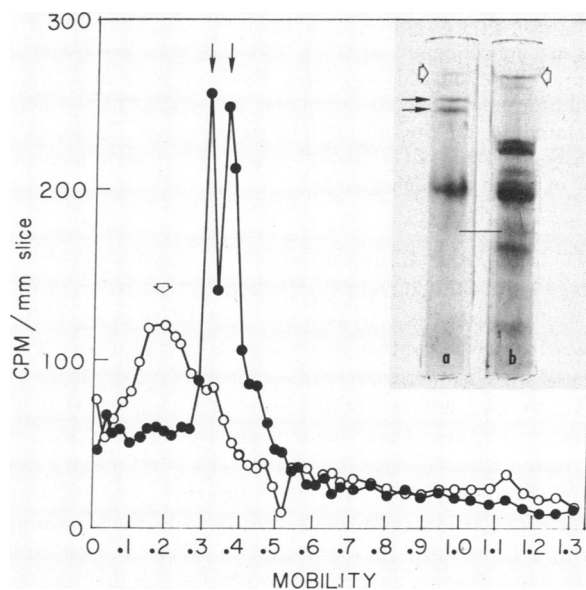


FIG. 5. Comparison of CSP and procollagen on NaDodSO₄ gels. Chick embryo fibroblasts were labeled with 2 μ Ci/ml of [¹⁴C]proline for 2 hr in serum-free medium containing 0.25 mM sodium ascorbate. The medium, which corresponds to the first extract during CSP isolation (see Table 1), was dialyzed, lyophilized, then resuspended in 0.5 mM CaCl₂, 50 mM Tris-HCl (pH 7.4). An aliquot was incubated with collagenase at 50 μ g/ml, 37°, for 5 min. Equal counts were placed on 5% cylindrical gels run in parallel. Inset: Gels stained for protein: (a) control; (b) collagenase-treated. Open arrow is the CSP. Closed arrows are the presumed $\alpha 1$ and $\alpha 2$ procollagen bands. Line indicates bromphenol blue fronts. Additional paired bands on collagenase gel due to collagenase; this gel contains more material because equal counts rather than equal amounts of protein were placed on the gels. Graph: [¹⁴C]proline labeling. (●) Control; (○) collagenase-treated. Arrows as in Inset.

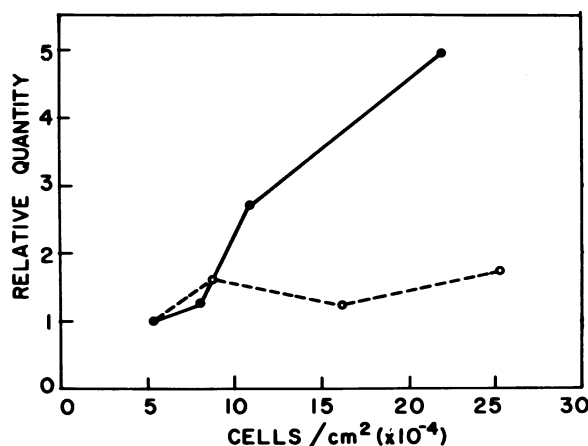


FIG. 6. Cell density effects on the quantity compared with the recovery of CSP. Chick embryo fibroblasts were plated at 2, 3, 4, and 6×10^6 cells per 100-mm dish and cultured 48 hr. CSP extracts were prepared from equal numbers of cells (10^7) at each density by adjusting numbers of dishes and volume of extract used after hemocytometer counts (>1000 cells counted per dish). The CSP extracts were subjected to electrophoresis, and absorbance of CSP peaks was determined by densitometry. All values are expressed relative to the amount of CSP recovered from the most sparse culture. NaDodSO₄ whole-cell homogenates were prepared from monolayers, and cell counts were performed on duplicate parallel cultures. Aliquots of homogenate equivalent to 3×10^6 cells were subjected to electrophoresis and measured by densitometry as above. At a density of 5×10^4 cells per cm², cells are subconfluent; at 25×10^4 /cm², cells are highly confluent, with numerous overlaps. Even at high densities, the absolute amount of CSP extracted was $<15\%$ of the amount estimated to be present in homogenates (see Table I). (●) Relative recovery of CSP by extraction; (○) relative quantity of CSP in whole-cell homogenates.

AMP levels, and stimulate mitosis in previously quiescent cells (10, 40–43). Conversely, the finding that recovery of this protein increases as cells become crowded (Fig. 6), may also relate to growth control, especially in light of evidence of increased membrane turnover in confluent cultures (44).

A definitive demonstration of this CSP's biological function, however, requires that removal and restoration of CSP be correlated with changes in cell behavior. Preliminary studies with ¹²⁵I-labeled, isolated CSP indicate that measurable quantities of intact protein can be adsorbed onto chick embryo fibroblasts (in preparation). Thus, it now appears possible to deplete (by proteolysis), isolate, and reattach CSP to cells. This potentially provides a direct approach to analysis of cell surface alterations during transformation.

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